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Production of a broad-specificity monoclonal antibody and application as a receptor to detection amatoxins in mushroom

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ABSTRACT

In this study, we report the production of a monoclonal broad-specificity monoclonal antibody (mAb) specific for amatoxins and development of an indirect competitive immunoassay for detection of amatoxins in mushroom samples. In the assay, the complete antigen (α-amanitin-OVA) was used as coating antigen, and amatoxins as competitor competes with coating antigen to bind with mAb. Using this approach, The half-maximum inhibition concentrations (IC_{50}) of α-amanitin, β-amanitin and γ-amanitin, and limits of detection (LODs, IC_{15}) were 66.3, 97.4, 163.1 ng/mL and 0.91, 0.98, 0.89 ng/mL, respectively. The LODs for α-amanitin, β-amanitin and γ-amanitin in mushroom samples were 4.55, 4.9, and 4.45 ng/mL. The spiked results were also confirmed by HPLC, which showed a good correlation ($R^2 = 0.996$) between the two methods. The results indicated that the developed assay was reliable and suitable for the detection of amatoxins in mushroom samples.

1. Introduction

In the past few decades, mushrooms have become popular in the human diet as a result of their exquisite taste and texture, protein content, and an expanding body of scientific research supporting their health benefits [1–3]. The increased public demand for wild edible mushrooms contributes to an increased interest in their picking and consumption [4], which enhances the risk of intoxications by toxic mushrooms [5]. Amatoxins, a group of bicyclic. With α-amanitin, β-amanitin and γ-amanitin being the main toxins in the high toxicity fungi. Amatoxin poisoning usually has a bad prognosis due to the high risk of liver failure. There are no worldwide widely accepted guidelines regarding the treatment of amatoxins-intoxicated patients and therapy comprises supportive care and numerous combinations of drugs. Thus, it is very important to develop rapid analytical methods for detection these toxins [6,7].

Currently, instrument-based methods, such as mass spectrometry [8], HPLC [9], electrochemical (EC) and LC–MS [10,11], are the most commonly used techniques for detecting amatoxins in different samples. However, these detection methods are very costly and time-consuming, and need professional personnel and expensive instruments. In comparison, immunoassays have received considerable attention as a simple, sensitive, cost-effective tool for high-throughput screening analyses in toxins monitoring programs for many years, and series of commercial ELISA kits have been available in many countries for many years [12,13]. However,. However, there is no report about ELISA method for amatoxins detection using polyclonal or monoclonal antibodies at home and abroad, and have no ELISA kit for amatoxins in the market by far.

Therefore, the present study aimed to produce and to characterize a specific monoclonal antibody against amatoxins, and an indirect competitive ELISA (ic-ELISA) were established for the detection of amatoxins based on this monoclonal antibody.

2. Materials and methods

2.1. Chemicals and reagents

Amatoxins were purchased from Beijing Puhuashi Technology Development Company (Beijing, China). 3,30,5,50-tetramethylbenzidine (TMB), N,N-dimethylformamide (DMF) 99.8%, complete and incomplete Freund's adjuvant, bovine serum albumin (BSA), ovalbumin (OVA), and poly(ethylene glycol) (PEG) 4000 were purchased from Sigma-Aldrich (Missouri, U.S.A.). Goat anti-mouse IgG (HRP) conjugate was purchased from Promega (Madison, U.S.A.). Hypoxanthineaminopterinthymidine (HAT), and hypoxanthinethymidine (HT) were from GIBCO, Invitrogen

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(California, USA.). Other reagents were chemical grade from Sinopharm Chemical Regent Co.Ltd. (Beijing, China). Female Balb/c mice were purchased from Wushi Animal Laboratory (Shanghai, China). Myeloma cells Myeloma cell SP2/0 were stored in our laboratory. Mouse antibodies isotyping kit was was purchased from Sigma Chemical Co. (St. Louis, MO). Phosphate-buffered saline (PBS) containing sodiumazide (0.01 M, pH 7.4) was used for diluting immunoreagents and samples. Phosphate-buffered saline with 0.05% Tween 20 (PBST) was for washing microtiter plates after each reaction step. Carbonate buffer containing sodium carbonate and sodium bicarbonate (0.05 M, pH 9.6) was used for coating coating-antigen on microtiter plates.

2.2. Synthesis of complete antigens

Complete antigens (α-amanitin-BSA) were prepared by two-step approach. Synthesis of α-amanitin-HS was produced by succinic anhydride method. A 20 fold molar excess of succinic anhydride solubilized in 150 μL of anhydrous pyridine was added to 1.5 mg powder of α-amanitin. After incubation 10 h at 70 °C, the solvent was evaporated at 50 °C for 30 min by rotary evaporation machine, and the residue was redissolved in 600 mL of DMF/H_2O (v:v, 6:9). Then the conjugates were stored at 4 °C for overnight. α-amanitin-HS was conjugated to carrier protein bovine serum albumin (BSA) using carbodiimide and Nhydroxysuccinimide. To 600 mL of DMF/H_2O (v:v, 6:9) containingα-amanitin-HS, 20 mL of carbodiimide hydrochloride solution (25 mg/mL in PBS) and 10 mLof N-hydroxysuccinimide solution (20 mg/mL in PBS) were added. The mixture was gently stirred at 25 °C for 1.5 h, and 0.25 mL of BSA or OVA solution (20 mg/mL in 0.05 M carbonate buffer, pH 9.6) was added to the mixture. After incubation for 1.5 h at 25 °C, the reaction mixture was dialyzed against PBS (0.01 M, pH 7.2) for 3 d at 4 °C, and then stored at −20 °C for usage [14,15].

2.3. Production of monoclonal antibody

Immunized 8-week–old BALB/c mice (Military Academy of Medical Science Beijing, China) were injected intraperitoneally into α-amanitin-BSA (100 μg in 0.25 mL PBS), mixed with an equal volume of FCA to form an emulsion. Subsequently, FIA substituted the FCA as the emulsifier and mice were injected with 100 μg of immunogen in booster injections every 3 weeks. After the third booster immunization, blood was collected from the mice tail vein, and the serum titer was determined to compete with α-amanitin, and continued until the serum titer was >1:8000. The mice were sacrificed 3 days later and their spleens were harvested. Fusion experiments were carried out as described previously. The positive hybridoma cells were subcloned by a limiting dilution method in the presence of thymocytes of Balb/c mice as feeder cells, according to standard protocols [16,17].

For monoclonal antibody production, 10 female Balb/c mice were used. The mice were treated by intraperitoneal injection with 0.5 ml of sterilized paraffin wax. After 10 days, each mouse was intraperitoneally injected with 1.6×10^6 hybridoma cells. The fluid was collected 7–10 days after hybridoma injection. Ascites fluid was initially purified by caprylic/ammoniunl sulfate precipitation [18].

2.4. Development of ELISA method

For optimization of the coating antigen, coating antigen α-amanitin–OA was used. The optimal dilutions of the coating antigens and antibody were determined by using the checkerboard procedure in which the wells with an absorbance of 1.0 were defined as the optimal coating antigen concentrations and antibody dilutions. Then, an indirect competitive ELISA (ic-ELISA) was developed as follows. Briefly, each well of a microtiter plate was coated with 100 μL of coating antigen, incubated overnight at 4 °C, and then blocked with 1% fetal calf serum. The plate was washed three times with PBST. Then, 50 μL of optimal antibody dilution and 50 μL of α-amanitin standard with series concentrations were added to the wells (in triplicate) for incubation for 1 h at 37 °C. The plate was washed as above. Then, 100 μL of HRP-conjugated goat anti-mouse IgG was added for incubation for 30 min at 37 μL. After washes, 100 μL of the TMB substrate system was added for incubation for 20 min at 37 °C. Finally, thereaction was stopped by the addition of 50 μL of 2 M H_2SO_4 to each well, and the plate was read on an ELISA plate reader at 450 nm to obtain the optical density (OD) values.

The cross-reactivity of the mAb was determined by ic-ELISA (ic-ELISA). We determined the average analyte concentration required for 50% inhibition (IC_{50}) and compared the values with the value from a standard curve for α-amanitin running on the same plate. Cross-reactivity was calculated as follows:

$$\text{Cross} - \text{reactivity (CR)}\ \% = \frac{\text{IC50(a} - \text{amanitin)}}{\text{IC50 (competitor)}} \times 100\%$$

2.5. Sample preparation and extraction

Mushroom samples were purchased from the Yonghui supermarket (Zhangjiakou, China). Amatoxin-free mushroom samples were attrited and homogenized in a homogenizer, and the homogenates were fortified with α-amanitin, β-amanitin, γ-amanitin to give the final concentrations at 5, 10, 20 ng/g.

For ELISA, 1 g amatoxin of mushroom sample was transferred into a 15 mL centrifuge tube and 4 mL of 50% (v/v) methanol solution was added. The sample was thoroughly vortexed for 1 h and centrifuged at 5000 × g for 30 min to deproteinate. The precipitate was dislodged, and the supernatant was then diluted with the 0.01 M PBS (pH 7.4) and then used for analysis.

For HPLC, 1 g amatoxin of mushroom sample was transferred into a 15 mL centrifuge tube and 4 mL of 50% (v/v) methanol solution was added. The sample was shaken on a vortex mixer for 1 h and centrifuged at 5000 × g for 30 min and the supernatant was passed through a filter (0.2 μm) prior to analy.

2.6. Recovery analysis in real samples

The amatoxin-free mushroom were minced and homogenized, and the homogenates were spiked with amatoxin to final concentrations of 5, 10, and 20 ng/g, respectively. The spiked samples were ncubated at 4 °C for 30 min. The samples were processed using the method described above. The extract was directly analyzed using ELISA, and the percentage recovery was calculated.

3. Results and discussion

3.1. Synthesis and analysis of complete antigen

α-amanitin is a low molecular weight hapten which is non-immunogenic, it needs to be conjugated with a carrier protein to stimulate animal response and produce antibody. In this study, bovine serum albumin (BSA) and ovalbumin (OVA) were used as carrier proteins. Complete antigens were prepared by chemical reaction method. The conjugations were identified using the method of UV scanning (Fig. 1A). The maximum absorption peak of α-amanitin, carrier protein, and α-amanitin-BSA were abvious different. The results indicated that the complete antigen were